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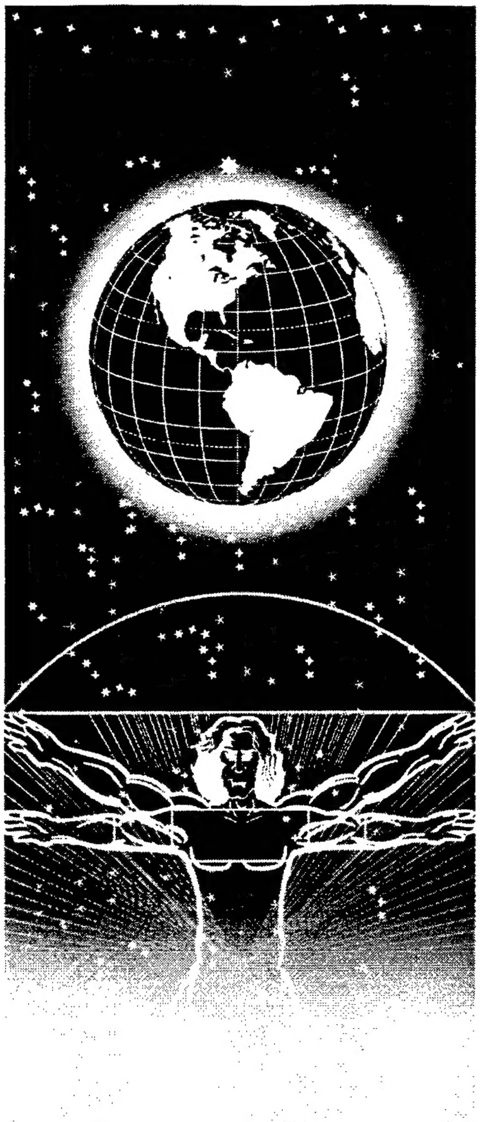

# UNITED STATES AIR FORCE ARMSTRONG LABORATORY

# In *Vitro* Effects of Ammonium Dinitramide


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March 1995

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The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

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**FOR THE COMMANDER**

Terry A. Childress

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Director, Toxicology Division
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Ammonium Dinitramide (ADN) is a high-energy compound under study as a replacement for current rocket propellants. This study determined the basic *in vitro* toxicity, stress gene induction, and genotoxicity of ADN. First, liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured to estimate membrane integrity of WB 344 hepatocytes. ADN increased leakage of both these enzymes at all concentrations studied. Normalized $EC_{50}$s were determined at percent of controls: ALT $EC_{50} = 2.7$mM and AST $EC_{50} = 3.2$mM. Next, to measure interactions of ADN with cellular regulatory transcription factors, genetically engineered human cell lines with fused human and bacterial stress-inducible genes were observed for the stress gene induction followed by ADN treatment. The stress reporter gene induction profile reflected that ADN induced the promoted sequences for all genes observed in the assay. Finally, assays to determine genotoxicity/mutagenicity of ADN were performed. These studies measured ADN's capability of damaging DNA, potentially giving rise to mutations and subsequent tumors. ADN exposed cells indicate that ADN has potential for directly affecting cellular DNA. This study is a vital part of the initial phase of toxicity testing and evaluation of ADN.

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## PREFACE

This report represents research performed by the Pharmacodynamics Group, Toxicology Division, Armstrong Laboratory, from October 1994 to March 1995. This project was supported by the Air Force Office of Scientific Research (AFOSR) project #2312A202.

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## INTRODUCTION

Ammonium dinitramide (ADN) is under consideration by the Department of Defense (DoD) and National Aeronautics and Space Administration (NASA)as a potential rocket propellant oxidizer. After further studies ADN may replace the current fuel ammonium perchlorate (AP)(Kinkead et al, 1994).

Studies from SRI International suggest that ADN may have the desirable qualities of clean burning, non detectability, and high performance. The oxidizer leaves no trail of hydrogen chloride in the exhaust plume, therefore eliminating environmental concerns about chlorine degradation of stratospheric ozone. ADN also reduces the smoke contrail thus reducing the chance of detection and tracking (Schmitt, 1990).

NASA has considered ADN as a high performance fuel additive to replace AP in the space shuttle booster rockets. ADN's better performance would mean more tonnage per launch into orbit (Schmitt, 1990). In order to study the basic toxicity of ADN we undertook several experiments.

The first studies examine selected toxicological effects in a rat liver cell line (WB344). The hepatocytes are used to investigate the viability endpoints after a 24 hour exposure to ADN. Analysis of intracellular enzyme leakage is used to determine the effective concentration to 50% maximum effect ($EC_{50}$), a standard expression of cytotoxicity.

A stress gene induction assay was performed to determine interactions of ADN with cellular regulatory transcription factors genetically engineered human cell lines with fused human and bacterial stress-inducible genes are observed for the stress gene induction. Induction is determined by observing for the transcription of specific reporter stress genes (Table 1) during the process of intracellular detoxification.

Finally assays to determine genotoxicity/mutagenicity of ADN were performed. These studies measured ADN's capability of damaging DNA, potentially giving rise to mutations and subsequent tumors. Recombination is the focus of this experiment by using yeast cells.

The results of these studies are vital parts of the initial phase of toxicity testing and evaluation of ADN. The goal is to better establish a model to determine exposure levels and health risks for DoD personnel working in the field.

## MATERIALS AND METHODS

### Test Chemical

The developer and supplier of ammonium dinitramide (ADN) $NH_4N(NO_2)_2$ for this study is SRI International, Menlo Park, CA.

### Cell Culture and Exposure Method on the WB 344 Hepatocytes

The WB 344 cell line was used in this study to measure cytotoxicity. The cell line is a diploid hepatic epithelial cells resembling the phenotype of mature hepatocytes in culture (DelRaso, 1992). The cell line was kept using culture in Corning 25 $cm^3$ tissue culture flasks with Minimum Essential Media (MEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc. Logan, UT) and 1% penicillin-streptomycin (Penn/Strep)(Gibco Laboratories, Grand Island, NY) All incubations were under a 5% $CO_2$ humidified incubator at $37^0$C.

Prior to exposure, the cells were trypsinize with 1X Trypsin EDTA (Gibco Laboratories, Grand Island, NY) and 5 x $10^5$ cells/ml were seeded in Falcon 6-well tissue culture plates (Becton Dickinson and Company, Lincoln Park, NJ). Four hours of preincubation time were allowed for the purpose of cell attachment.

After cell attachment treated cells were exposed to medium containing: 0mM, 0.25mM, 0.5mM, 1.0mM, 1.25mM, 1.50mM, 2.0mM, 2.5mM, 3.0mM, 3.5mM, 4.0mM, 6.0mM, 8.0mM, 10.0mM. Each concentration was done in triplicate. After 24 hours, media samples were collected in microcentrifuge tube for analysis of enzyme leakage. Total AST and ALT were determined by collecting all cells and analyzing the cell homogenate on the Kodak Ektachem 700XR Analyzer. To determine the $EC_{50}$ the data was normalized to fit a scale of O% to 100% effect. Data normalization was calculated by using the equation:

%Effected = [(X%-Rmin)/Rmax-Rmin)]*100%

**X%= raw data**

**Rmin=minimum leakage**

**Rmax=maximum leakage**

## Genotoxicity

A yeast cell strain XY2 which is isogenic to the RS112 strain with the deletion of the excision repair gene, RAD2, was used in this study to determine genotoxicity (Sommers et al, 1995). The cells were exposed to ADN at concentrations of 0mM, 2.5mM, 5.0mM, 10.0mM, 100mM, 500mM, and 1000mM. The Yeast DEL Assay(Xenometrix, Inc, Boulder, CO) is a short term assay in microtiter form that allow for more accurate quantitation. In addition, the Yeast DEL Assay detects chemicals known to be carcinogens, which the Ames Assay does not(Sommers et al, 1995). Studies by Sommers, et al show methanesulfonic acid methyl ester (Sigma Chemical Co, St. Louis, MO), a known carcinogen, tests positive for recombination in the Yeast DEL Assay at concentrations greater than 33ug/ml. Consequently, 0.454mM of methanesulfonic acid methyl ester was used as a positive control.

To validate the assay cytotoxicity was assured by using a range of ADN concentrations known to produce a viability of 20% or greater. One may expect that chemicals which are not carcinogenic may cause recombination at cytotoxic levels. Therefore, the results are only significant when the viability is greater than 20%. Positive responses are indicated when there is an increase in DNA recombination frequency greater than three time the control recombinant frequency. Results are reported as fold induction of recombination frequency.

## Induction of Stress Genes

The CAT-Tox (L) Assay (Xenometrix, Inc. Boulder, CO) uses genetically engineered reporter constructs of specific stress gene promoter regions. Human (HepG2) Liver cells are transfected by 14 separate promoter fusions (Table 1) and a wild type human liver cell line is used to check cell viability. Cells were dosed with ADN at 0mM, 10mM, 30mM, 40mM, 50mM, and 75mM. Concentration were determine by the results of Pre-Cat(L) Assay (Xenometrix, Inc. Boulder, CO) which determines viability of the cell culture, HepG2.

After washing, cells were lysed and analyzed for protein content for determination of the specific reporter protein, chloroamphemical acetyl transferrin (CAT), expressed by the promoter

fusion constructs. Viability was determined from the wild type human liver cells with the same ADN concentrations as the test assay.

| GENE | INDUCERS |
|---|---|
| CYP1A1 | aromatic hydrocarbons |
| GST Ya | electrophile, alkylating |
| XRE | aromatic hydrocarbons |
| MT IIA | heavy metals |
| FOS | c-AMP, $CA^{2+}$, arachidonate |
| NFkBRE | mitogens, thiols |
| XHF | mitogens inflammation |
| HSP70 | heat shock protein 70, protein denaturants |
| CRE | cyclic AMP |
| p53RE | DNA damaging agents |
| RARE | retinoic acid and its analogs |
| GADD153 | growth arrest, DNA damage |
| GADD45 | growth arrest, DNA damage |
| GRP78 | calcium ionophore, thapsigargin, and DNA damaging agents |

**Table 1**. Gene promoter fusion constructs. These are transfected into human cell line (HepG2). Induction of the genes is measured by assay for the reporter CAT protein.

## RESULTS

### Enzyme leakage assay

Enzyme leakage assays were performed on the WB 344 cell culture to determine the levels of the ADN that will effect the cell membrane integrity and to quantitate the $EC_{50}$ in this particular cell line. Measurement of AST and ALT leakage is a standard endpoint for determining cell membrane integrity (Pravecek et al, 1994). Enzyme leakage was linear with respect to ADN exposure concentration. For AST leakage the $EC_{50}$ was 3.2mM and for ALT leakage it was 2.7mM (Figure 1A and 1B). These data suggest that ADN at concentrations less than the $EC_{50}$ value do not significantly affect the cell membrane.

**Genotoxicity**

| Concentration (mM) | Percent Survival | standard deviation | Recombination Frequency | standard deviation | Fold Induction |
|---|---|---|---|---|---|
| 0.00 | 100.00% | ±[GU1]0.00 | 4.14e-4 | ±3.70e-5 | - |
| 2.50 | 102.05% | ±14.81 | 4.17e-4 | ±5.09e-5 | 1.01 |
| 5.00 | 61.87% | ±11.63 | 5.39e-4 | ±1.19e-4 | 1.30 |
| 10.00 | 53.04% | ±6.08 | 5.33e-4 | ±7.80e-5 | 1.29 |
| 100.00 | 48.58% | ±6.41 | 5.61e-4 | ±1.31e-4 | 1.36 |
| 500.00 | 15.28% | ±3.83 | 1.44e-3 | ±5.64e-4 | 3.47 |
| 1000.00 | 0.00% | ±0.00 | 0.00 | ±0.00 | 0.00 |
| +control(Methanesulfonic acid methyl ester) 0.45mM | 57.48% | ±6.48 | 1.62e-3 | ±3.25e-4 | 3.91 |

**Table 2.** The genotoxicity result indicate no fold induction of three or greater except at cytotoxic level of 500mM concentration and on the positive control.

Results from the genotoxicity assay indicate that ADN has potential for directly affecting cellular DNA (Table 2). Levels greater than 10 mM ADN were clearly cytotoxic as seen in (Figures 2). There was a linear decrease in cell viability with increasing ADN concentration. There is a 3.47 fold induction in recombination at 500mM ADN, however at this concentration viability decreased to 15.3%.

**Induction of Stress Genes**

| 30mM | 40mM | 50mM | 75mM |
|---|---|---|---|
| GST Ya, FOS | CYP1A1, GST Ya, XRE, HMTIIA, NFkBRE, XHF, HSP70 | CYP1A1, GST Ya, XRE, HMTIIA, FOS, NFkBRE, XHF, HSP70, RARE | CYP1A1, GST Ya, XRE, HMTIIA, FOS, NFkBRE, XHF, HSP70, p53RE, RARE, GADD153, GRP78 |

**Table 3.** Promoter gene constructs that were induced.

The stress reporter gene induction profile suggested the mechanism of toxicity for ADN. ADN induced the promoter sequences for all genes listed on Table 3. However, these were induced significantly only at ADN concentrations that may compromise the viability of the cells seen in Figure 3 and 4.

**DISCUSSION**

The results of the enzyme leakage assay indicates that ADN concentrations greater than 2.7mM damages the cell membrane integrity of 50% of the cells. Comparison with hydrazine hydrate, where AST levels were not affected at 1.2mM, 2.4mM, 5.0mM. (Pravecek et al, 1994). This suggest ADN is more potent in causing cellular membrane toxicity.

The results of the Yeast recombination assay suggest that ADN may be a genotoxin. The assay uses two criteria, survival of the yeast cells and the recombination frequency. Significant recombination occurred only at 500mM ADN, however at this level the viability of the yeast is only 15.28% ±3.83. This indicates that ADN may not be direct acting genotoxin. Rather, it would suggest that ADN is a cytotoxic genotoxin; i.e. significant injury to the cell must occur before DNA is affected. ADN does not have the potency of methanesulfonic acid methyl ester (positive control), which causes DNA recombination at the concentration of 0.454mM without reducing cell viability. DNA recombination occurs when ADN concentration reaches approximately a thousand times greater than methanesulfonic acid methyl ester.

The stress gene induction assay results show that ADN induces promoter stress genes GST Ya and FOS at 30mM. All genes which have been induced at concentration higher than 30mM are assumed to be the consequence of cell destruction because induction occurs at cytotoxic levels of ADN. These false positives should not be misinterpreted. However, 30mM of ADN causes a greater than two fold induction of GST Ya and FOS(2.60 and 3.50 fold induction, respectively) with corresponding viability of 64.3%. The cell line with the promoter GST Ya responds to PAHs, and phenolic antioxidant. The cell line with the promoter FOS responds to mitogens, DNA damaging agents and also to heat shock. This suggest that ADN may act through an oxidative challenge which may directly, or indirectly damage nuclear DNA. The results of this study give the groundwork of the toxicity testing for future follow up investigations for ADN.

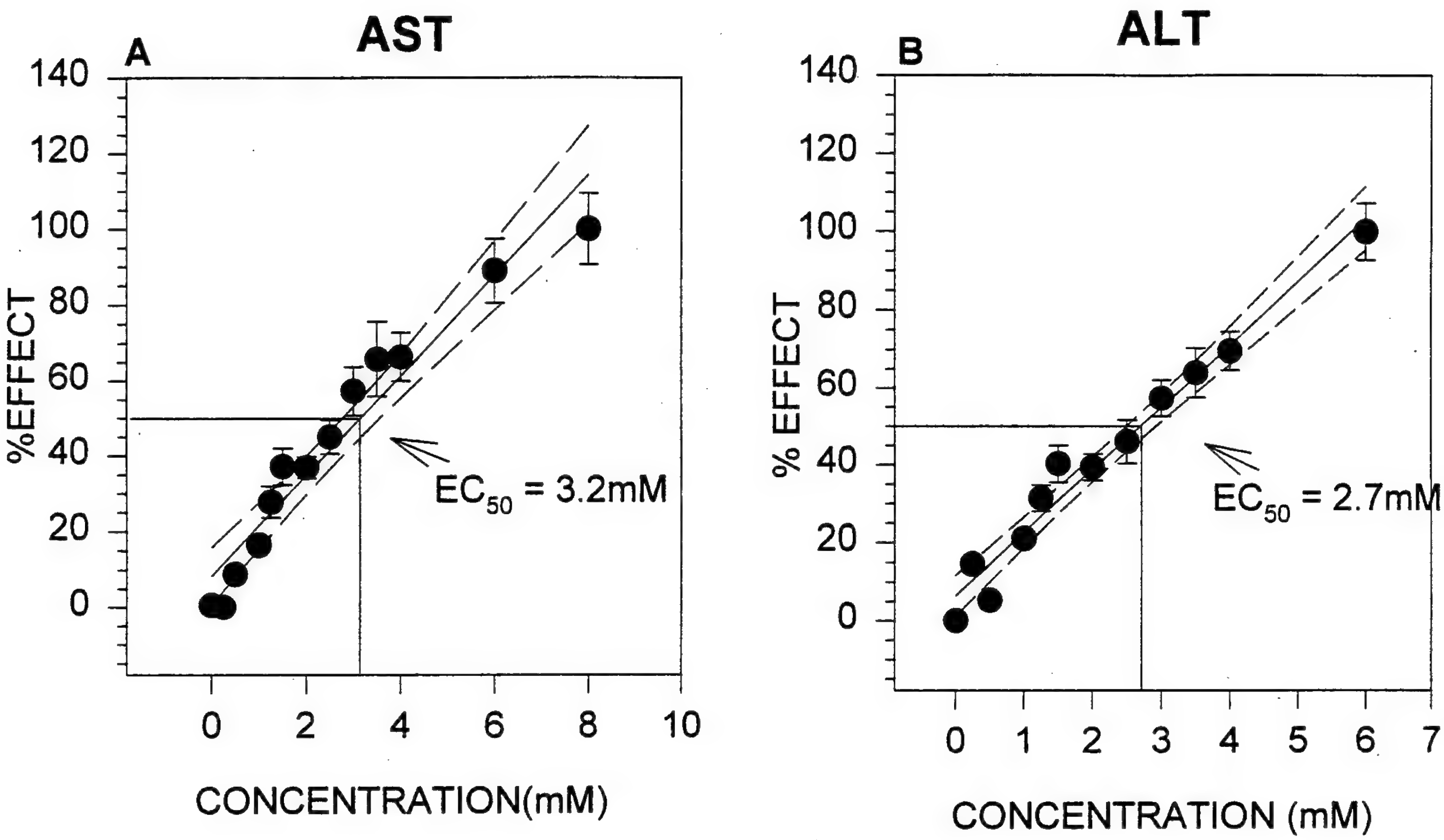


**Figure-1A. and Figure-B. AST and ALT % effect. The graph of Figure-1A** represents the % effect of AST. The $EC_{50}$ for AST is 3.2mM of ADN. Figure-1B represent the ALT exposure. The $EC_{50}$ for the ALT is 2.7mM ADN

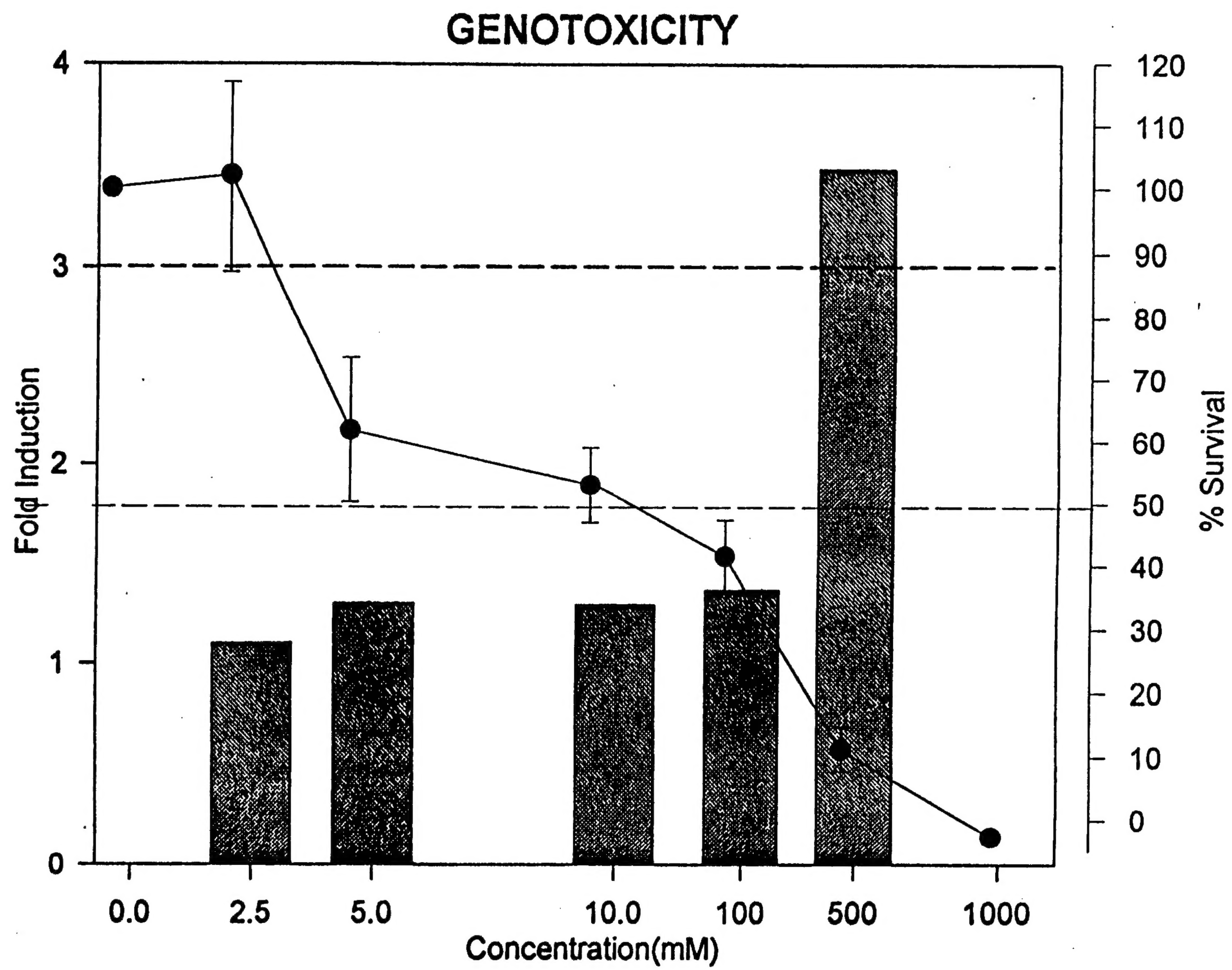


**Figure-2. Genotoxicity result.** The results show significant fold induction at 500mM and note the decrease in % survival as induction increase.

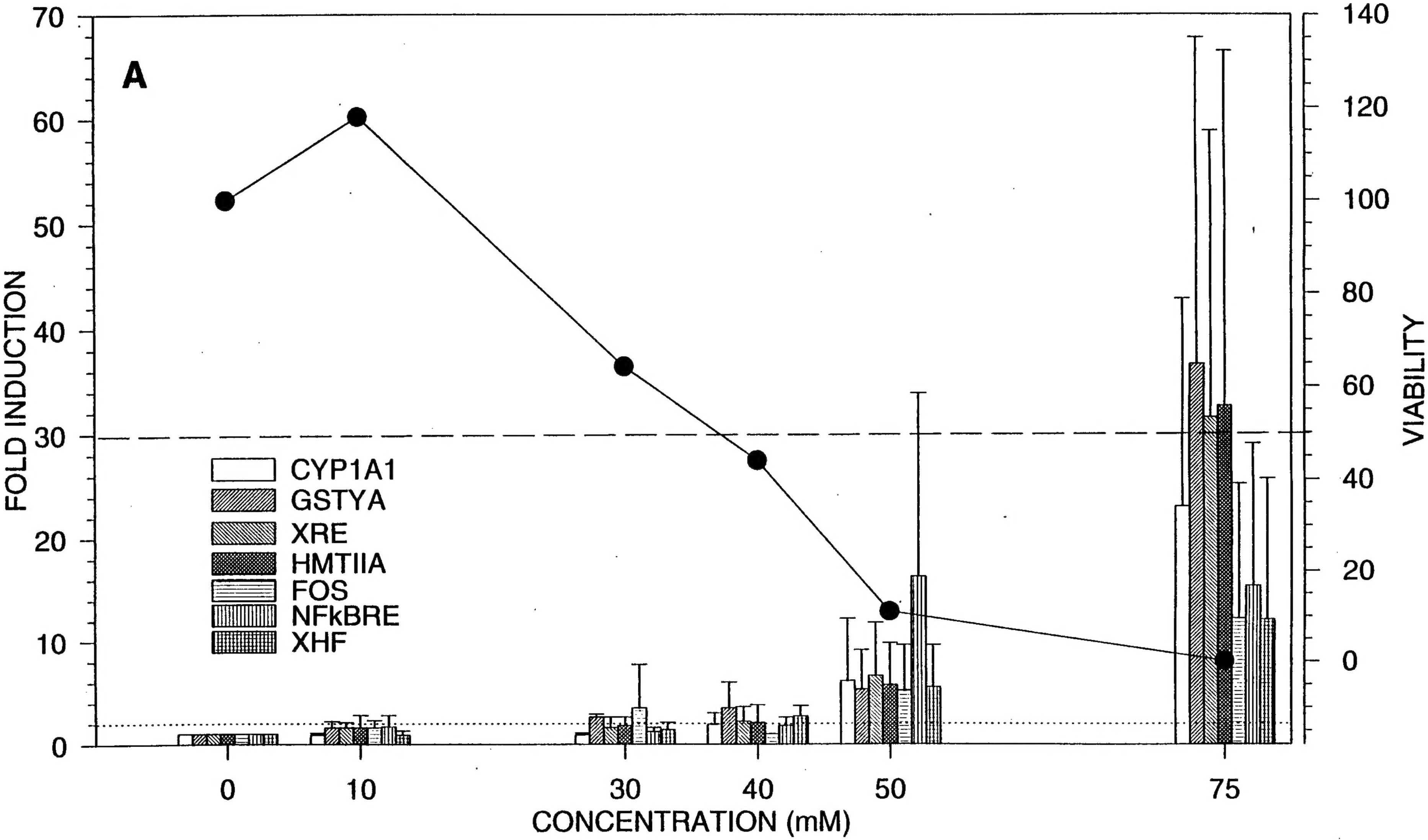


**Figure-3.** Shows the results of the CAT-Tox (L) Assay. The specific fusion genes are indicated by the legends. Induction are at a significant levels when ADN is at cytotoxic concentration.

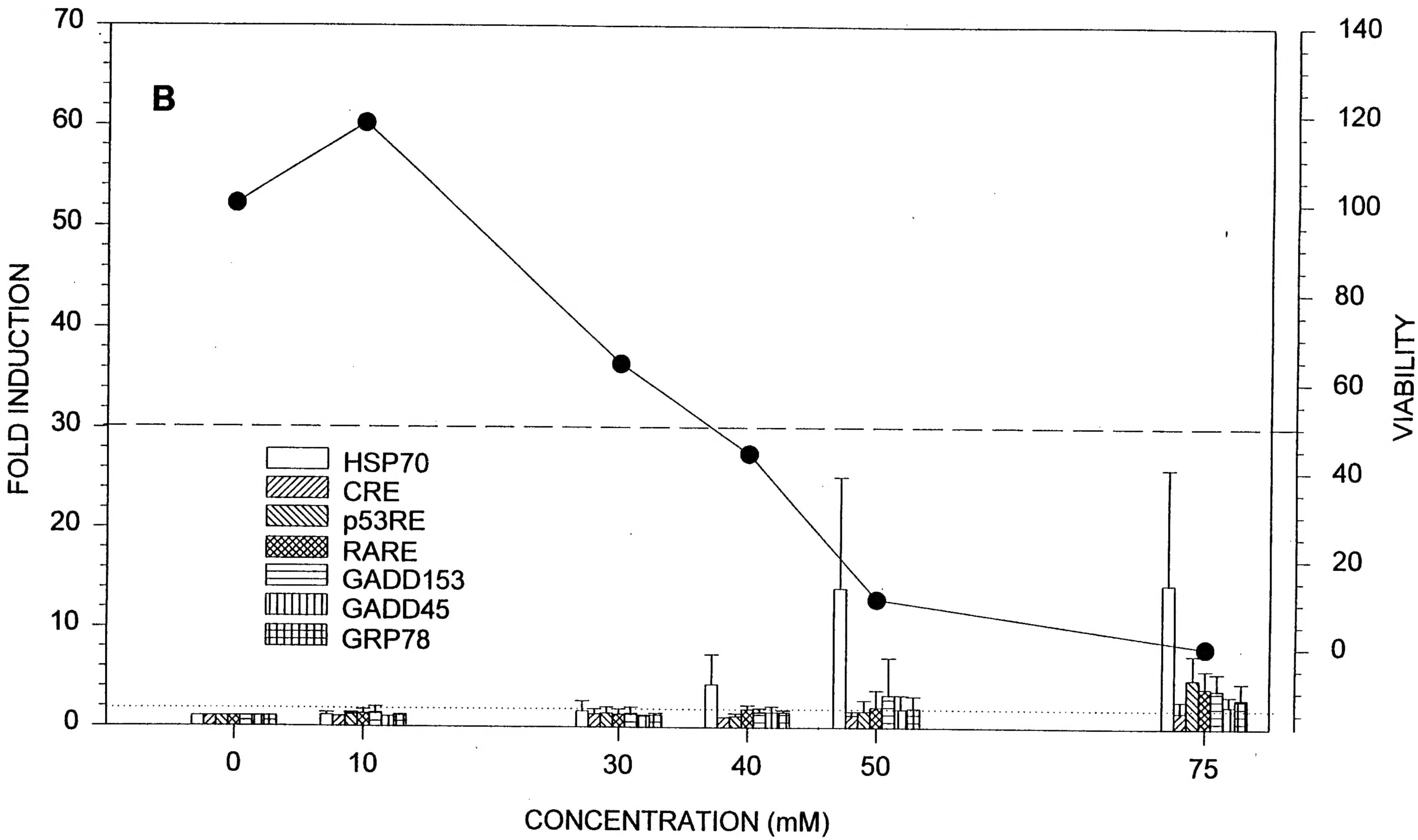


**Figure-4.** Shows induction of the specific fusion gene reporting for the CAT protein. The cell line do not appear to be effected until the level in concentration become cytotoxic to the cells. The viability is at less that 50% during significant fold induction.

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